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Effect of *Allium sativum* and Fish Collagen on the Proteolytic and Angiotensin-I Converting Enzyme-inhibitory Activities in Cheese and Yogurt

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Abstract: There is an increasing demand of functional foods in developed countries. Yogurt plays an important role in the management of blood pressure. Several bioactive peptides isolated from *Allium sativum* or fish collagen have shown antihypertensive activity. Thus, in the present study the effects of *A. sativum* and/or Fish Collagen (FC) on proteolysis and ACE inhibitory activity in yogurt (0, 7 and 14 day) and cheese (0, 14 and 28 day) were investigated. Proteolytic activities were the highest on day 7 of refrigerated storage in *A. sativum*-FC-yogurt (337.0±5.3 μg g^{-1}) followed by FC-yogurt (275.3±2.0 μg g^{-1}), *A. sativum*-yogurt (245.8±4.2 μg g^{-1}) and plain-yogurt (40.4±1.2 μg g^{-1}). On the other hand, proteolytic activities in cheese ripening were the highest ($p<0.05$) on day 14 of storage for plain and *A. sativum*-cheeses (411.4±4.3 and 528.7±1.6 μg g^{-1}, respectively). However, the presence of FC increased the proteolysis to the highest level on day 28 of storage for FC- and *A. sativum*-FC cheeses (641.2±0.1 and 1128.4±4.5 μg g^{-1}, respectively). In addition, plain- and *A. sativum*-yogurts with or without FC showed maximal inhibition of ACE on day 7 of storage. Fresh plain- and *A. sativum*-cheeses showed ACE inhibition (72.3±7.8 and 50.4±1.6 % respectively), the presence of FC in both type of cheeses reduced the ACE inhibition to 62.9±0.8 and 44.5±5.0 %, respectively. However, refrigerated storage increased ACE inhibition in cheeses ($p<0.05$ on day 28) in the presence of FC more than in the absence. In conclusion, the presence of FC in *A. sativum*- yogurt or cheese enhanced the proteolytic activity. Thus, it has potential in the development of an effective dietary strategy for hypertension associated cardiovascular diseases.

Key words: Yogurt, cheese, fish collagen, *Allium sativum,* angiotensin-1 converting enzyme

INTRODUCTION

Hypertension is one of the most common cardiovascular diseases. It is a worldwide problem of epidemic proportions, which affects 15-50% of all adults. It is the most common serious chronic health problem because it carries a high risk factor for arteriosclerosis, stroke, myocardial infarction and end-stage renal disease (Ranade *et al*., 2001). Therefore, the role of the Rennin-angiotensin System (RAS) in cardiovascular physiology is well established.

The angiotensin-I converting enzyme (ACE), a component of RAS catalyzes the formation of the strong pressor agent angiotensin II from angiotensin I contributing to the maintenance of normal blood pressure (Coates, 2003; Unger, 2002). Captopril, enalapril, lisinopril and temocapril are an ACE inhibitors drug used to treat hypertension. All of these drugs produced side effects thus, justifying the search for natural ACE inhibitors for safe and economical use (Coates, 2003; Kang *et al*., 2003). The demand for functional foods has been boosted in recent years as a result of growing awareness among consumers of the link between diet and health (FitzGerald *et al*., 2004). The consumption of dairy food such as cheeses and yogurt has increased rapidly owing to the fact that these products fulfill many of the current dietary needs. In addition, the proteolysis of milk protein which takes place during fermentation is a key metabolic activity to supply nitrogenous compounds for microbial growth (Tamime and Robinson, 1999). This makes the consumption of fermented milk products are associated with the reduction of blood pressure (Hernadez-Ledesma *et al*., 2003). However, the mechanism of action based on the liberation of peptides during proteolysis activity of Lactic Acid Bacteria (LAB) which causes inhibition of angiotensin-I converting enzyme (Fuglsang *et al*., 2003; Ijas *et al*., 2004; Vermeirssen *et al*., 2002). Many peptides with antihypertensive action have

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been characterized upon fermentation of milk with different microorganisms or by the action of pure proteinases on milk proteins (Abubakar *et al*., 1998; Hernandez-Ledesma *et al*., 2002; Maeno *et al*., 1996; Tauzin *et al*., 2002). The degradation of milk proteins with proteinases from *Lactobacillus helveticus* produced peptides with ACE-inhibiting activity which has a significant antihypertensive effect *in vivo* (Yamamoto *et al*., 1994) and *in vitro* (Nakamura *et al*., 1995). In addition, water soluble extracts of different cheese varieties such as Norvegia, Jarlsberg, Cheddar and Blue (Stepaniak *et al*., 2001) or Gouda, Emmental, Blue, Camembert, Edam and Havarti (Saito *et al*., 2000) have been shown to possess ACE-inhibitory activity *in vitro*. Moreover, yogurt in the presence of water extract from some medicinal herbs such as *Azadirachta indica* (Shori and Baba, 2011a) or *Mentha piperita*, *Anethum graveolence* and *Ocimum basilicum* (Amirdivani and Baba, 2011) shows to inhibit ACE enzymes during storage at 4°C up to 28 days.

A number of compounds from plants have been identified to possess in vitro ACE inhibitory activity including hydrolysable tannins, phenylpropanes, proanthocyanidins, flavonoids, xanthones, fatty acids, terpenoids, alkaloids oligosaccharides and peptide amino acids (Park *et al*., 2003; Nyman *et al*., 1998). Garlic (*Allium sativum*) has beneficial effects on blood pressure because it eases the spasm of the small arteries, slows the pulse and modifies the heart rhythm (Silagy and Neil, 1994). In addition, bioactive peptides isolated from fish collagen extracted from bones and scales of fish have shown antihypertensive activity inhibiting the action of ACE (Kim *et al*., 2000). These peptides found to be stronger than that of many other natural peptides (Kim *et al*., 2000) and showed ability to reduce blood pressure in spontaneously hypertensive rats *in vivo* (Je *et al*., 2005; Fujita and Yoshikawa, 1999). Therefore, the purpose of the present study is to investigate the effects of inclusion of *A. sativum* and/or Fish Collagen (FC) in yogurt and cheese on milk protein proteolysis during refrigerated storage and the ability of their fermented milk products to inhibit ACE activity *in vitro*.

MATERIALS AND METHODS

Materials and chemicals: Pasteurized fresh full cream cow milk and *A. sativum* powder (McCormick®) were purchased from convenient store. Fish collagen and yogurt bacteria mixture (Chris-Hansen, Denmark) contains *Lactobacillus acidophilus* LA-5 (4 billion), *Bifidobacterium* Bb-12 (4 billion), *Lactobacillus casei* LC-01 (1 billion) and *Streptococcus thermophilus* Th-4 (1 billion) and *L. bulgaricus* in the ratio of 4:4:1:1:1 were purchased through local supplier. All chemicals used in the present study were of analytical grade and were purchased from (Sigma Chemical Co. (USA).

Herbal water extract: *A. sativum* powder (5 g) was homogenized (Polytron, 10 sec at maximum setting) in sterile distilled water and the volume was made up to 50 mL. The homogenate was kept in a water bath (70°C) for 12 h followed by centrifugation (2000 rpm, 4°C, 15 min). The supernatant was harvested and kept refrigerated (4°C) and used in the making of yogurt and cheese within 24 h.

Yogurt preparation: Firstly, yogurt starter culture was prepared by adding yogurt bacteria mixture into pre-heated pasteurized fresh full cream milk (41°C, 1L) and these were mixed thoroughly prior to 12 h incubation at 41°C. The yogurt starter culture was kept refrigerated (4°C) and used within 3 days. Later, pasteurized fresh full cream milk (850 mL) was initially heated to 41°C. Yogurt starter culture (50 g) was added (Shah, 2003) followed by the addition of 100 mL *A. sativum* water extract and 2.0 g of full cream milk powder to correct the solid content. The mixture was thoroughly mixed and then aliquoted (100 mL) into disposable containers. These were placed in an incubator at 41°C and the fermentation process was terminated at pH 4.5 by placing the container in ice-bath for 60 minutes. Yogurts were then kept in refrigerator (4°C) for predetermined periods prior to analysis. The same procedures were carried out to prepare plain yogurt except that 100 mL of distilled water was used in place of *A. sativum* water extract. *A. sativum*- and plain-yogurt in presence of FC were prepared by addition of FC powder (2.5% w/v) to each yogurt prior to incubation at 41°C.

Cheese preparation: Four types of cheeses i.e., plain cheese, *A. sativum* cheese, plain cheese with FC and *A. sativum* cheese with FC were made. These cheeses were either unripened (fresh cheese) or ripened for 14 and 28 days in a refrigerator (8°C). The cheese was prepared as follows: fresh cow's milk (4 L) was pasteurized by heating to 72°C for 15 sec followed by cooling to 32°C in an ice bath. Yogurt starter culture (200 mL) was added into 4 L pasteurized milk and the mixture was stirred thoroughly and incubated in the water bath (41°C) for 1 h. The inoculated milk was then divided equally into four beakers and 2 mL of rennet solution (one tablet of Danisco Marcshall™ M-50 microbial rennet dissolved in 100 mL of distilled water)

was added to each beaker followed by gentle stirring for 5 min. The curdling process was allowed to take place at 32°C in water bath for five hours. When the curd was set, the coagulant was cut using a knife into grain size. The curd was then gently stirred until the first flush of whey has left the curd particles. The whey was drained off and *A. sativum*-water extract (3 mL) without or with 2.5 g FC powder was mixed with the curd to prepare *A. sativum*- and *A. sativum*-FC cheeses, respectively. Plain cheese without or with 2.5 g FC powder was prepared in the same way except that 3 mL of distilled water were added instead of 3 mL *A. sativum* water extract. The curd was placed into clean Muslin cloth which was subsequently placed in a mould. The fresh cheese formed was mechanically pressed by placing a 2 kg weight for 6 h in the refrigerator (8°C). All pressed cheeses were salted in 6% brine for 8 h. The cheeses were then placed in a porous container and ripened in the refrigerator (8°C) for predetermined periods (14 and 28 days).

Preparation of yogurt water extracts: The yogurt water extract was prepared as described by Shori and Baba (2011a).

Preparation of cheese water extracts: The cheese water extract was prepared according to Pripp *et al.* (2006).

Determination of proteolytic activity: Proteolytic activities in yogurt and cheese were assessed by measuring liberated amino acids and peptides using the o-phthaldialdehyde (OPA) based spectrophotometric method according to Church *et al.* (1983).

Determination of ACE inhibitory activity: ACE was extracted from fresh rabbit lung by grinding rabbit lung using pestle and mortar with ice-cold 50 mmol L^{-1} Tris-HCl with 400 mmol L^{-1} NaCl, pH 8.3 (Vermeirssen *et al.*, 2002). ACE inhibitory activity was assayed by mixing ACE reagent (500 μL; 1.0 mmol L^{-1} Furanacryloyl-Phe-Gly-Gly (FAPGG) in 50 mmol L^{-1} Tris-HCl with 400 mmol L^{-1} NaCl, pH 8.3) with 300 μL of yogurt/cheese water extract in a cuvette. The mixture was mixed thoroughly and incubated in a water bath (37°C) for 2 min. This was followed by addition of 300 μL of rabbit lung enzyme in 50 mmol L^{-1} Tris-HCl, pH 8.3 and the mixture was mixed carefully. Absorbance was measured at 340 nm and the decrease in the enzymatic reaction was monitored for a total period of 20 min with brief absorbance reading every 5 min. Inhibition effects on the enzyme activity by test samples were represented as % of inhibition which calculated as follows:

$$\text{ACE-inhibition (\%)} = \frac{1-(C-D)}{(A-B)} \times 100$$

where, A is the absorbance in the presence of ACE and without the sample, B is the absorbance without both ACE and the sample, C is the absorbance with ACE and the sample and D is the absorbance with the sample but without ACE.

Statistical analysis: Experiments were carried out on yogurt and cheese prepared in three different batches (3x3). Analysis was performed using SPSS software. Data are presented as Mean±SEM. One-way analysis of variance (ANOVA) was used and the differences between the treatment means were compared at $p = 0.05$.

RESULTS

Our results observed that, the presence of *A. sativum* in yogurt and cheese provides a relatively positive effect on proteolysis activity of milk protein. However, addition of fish collagen in both yogurt and cheese had significant influence on the proteolytic activity. Refrigerated storage enhanced inhibitory activity of ACE more in yogurt than cheese after inclusion of *A. sativum* and/or fish collagen.

The OPA-based spectrophotometric method based on detection of released amino groups resulting from the proteolysis of milk proteins which gave a direct measurement of proteolytic activity. Fresh plain yogurt (0 day) showed proteolytic activity (23.6±0.1 μg g^{-1}) afterward small changes in proteolytic activity were occurred during storage (Fig. 1). However, the presence of *A. sativum* increased ($p>0.05$) the proteolysis to 33.9±0.4 μg g^{-1} at 0 day of storage. Refrigerated storage of *A. sativum*-yogurt to 7 days resulted in an increase in proteolysis to 245.8±0.2 μg g^{-1} followed by a decrease to 34.3±0.1 μg g^{-1} on day 14 of storage (Fig. 1). The addition of FC in fresh yogurt with or without *A. sativum* water extract showed noticeable increased in proteolytic activity ($p<0.05$) to 303.3±0.3 μg g^{-1} and 181.2±0.1 μg g^{-1}, respectively (Fig. 1). The extent of proteolysis increased more in FC-yogurt (275.3±1.0 μg g^{-1}) than in *A. sativum*-FC yogurt (337.0±0.5 μg g^{-1}) during 7 days of storage. However, prolonged storage to 14 days causes reduction in proteolytic activity to 290.8±0.8 and 199.8±0.8 μg g^{-1} for *A. sativum*-FC yogurt and FC-yogurt, respectively. On the other hand, proteolytic activity in fresh cheeses (0 day) ranged from 110 to 330 μg g^{-1} (Fig. 2). Refrigerated storage to 14 days increased proteolysis activity for all cheeses with the highest increase was observed in *A. sativum*-FC cheese (1073.3±0.2 μg g^{-1})

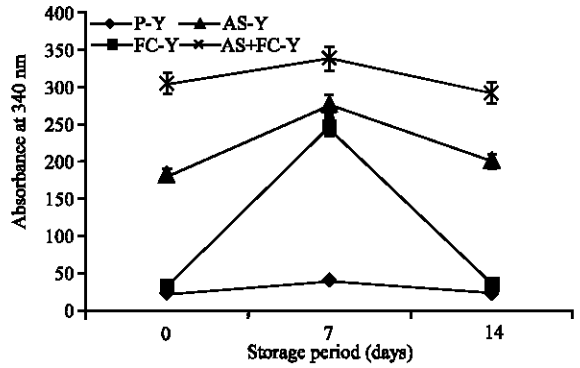

Fig. 1: Proteolytic activity of yogurt during storage at 4°C, P: Plain, Y: Yogurt, AS: *A. sativum*, FC: Fish collagen, values are presented as Mean±SEM (n = 3)

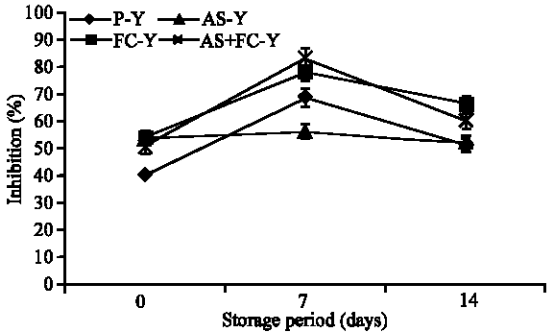

Fig. 3: Changes in ACE inhibition activity (%) in yogurt during storage at 4°C, P: Plain, Y: Yogurt, AS: *A. sativum*, FC: Fish collagen, values are presented as Mean±SEM (n = 3)

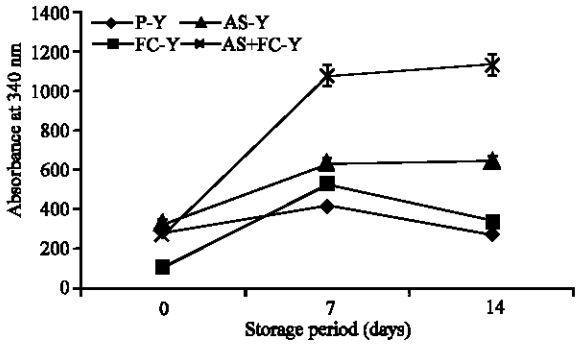

Fig. 2: Proteolytic activity of cheese during ripening at 8°C. P: Plain, CH: Cheese, AS: *A. sativum*, FC: Fish collagen, values are presented as Mean±SEM (n = 3)

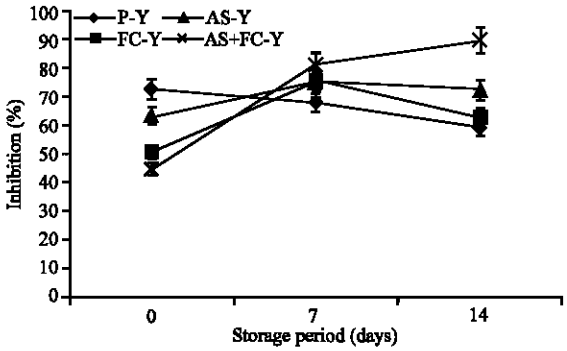

Fig. 4: Changes in ACE inhibition activity (%) in cheese during ripening at 8°C, P: Plain, CH: Cheese, AS: *A. sativum*, FC: Fish collagen, values are presented as Mean±SEM (n = 3)

followed by FC- cheese (629.9±0.6 μg g^{-1}), *A. sativum*-cheese (528.7±0.7 μg g^{-1}) and plain-cheese (411.4±0.33 μg g^{-1}). The proteolytic activity had minimal increase in *A. sativum*-FC cheese (1128.4±0.6 μg g^{-1}) and FC-cheese (641.2±0.2 μg g^{-1}) with extended storage to 28 days whereas *A. sativum*- and plain-cheeses reduced to 339.8±0.5 and 274.9±0.9 μg g^{-1}, respectively (Fig. 2).

ACE inhibitory activities: Plain yogurt showed the lowest inhibition on ACE activity (40.12±0.6) on day 0 whereas *A. sativum*-yogurt, FC-yogurt and *A. sativum*-FC yogurt had higher ($p<0.05$) ACE inhibition activities (50-55%; Fig. 3). All yogurts, except FC-yogurt showed increased in ACE inhibition activities after 7 days of refrigerated storage. *A. sativum*-and *A. sativum*-FC yogurts inhibited ~80% of ACE activities (77.8±2.0 and 82.5±1.4%, respectively) which were higher ($p<0.05$) than plain- and FC-yogurts (68.4±2.6 and 55.9±1.5%, respectively). In comparison to yogurt, fresh cheeses (Fig. 4) showed significant differences in ACE inhibition activities with plain cheese showing the highest inhibition (72.4±1.9%) followed by FC-cheese, *A. sativum*-cheese and *A. sativum*-FC cheese (62.9±0.8, 50.4±1.6 and 44.6±5.0%, respectively). All cheeses, except plain cheese, showed increased in ACE inhibition activities (75-80%; $p<0.05$) after 14 days ripening at 8°C. In addition, ripening of cheeses to 28 days increased *A. sativum*-FC cheese ACE inhibition further to 89.3±2.6% whereas plain-FC-and *A. sativum*-cheeses had reduced inhibition of ACE activities (58.9±2.0, 71.9±1.8 and 62.7±1.8%, respectively).

DISCUSSION

Proteolysis of milk protein during fermentation of yogurt and ripening of cheese is a favorable process because it increases the milk protein digestibility (Lee *et al*., 1988). In the present studies *A. sativum* and/or FC increased proteolysis in fresh yogurt but not in fresh

cheese. This could be explain by the relatively longer fermentation step (4 h at 41°C) for yogurt preparation which may enhanced microbial proteolysis of milk protein compared to cheese making (1 h at 41°C). The present study showed remarkable effect on proteolysis activity during refrigerated storage which is in agreement with other studies such as Shori and Baba (2011a-c) for herbal yogurt and Andic *et al*. (2010) for Motal cheese. Increase proteolysis in the presence of FC, *A. sativum* and combination of the two implicated to formation of bioactive peptides. Previous studies reported that, the fortification of yogurt with fruits and other plant extracts profoundly change yogurt properties i.e., microbial content (Behrad *et al*., 2009; Shori and Baba, 2012) and even enhance the therapeutical values (Zainoldin and Baba, 2009; Behrad *et al*., 2009; Shori and Baba, 2011a-c; Amirdivani and Baba, 2011). Fermented milk products reported to contain bioactive peptides act as ACE inhibitor (Nakamura *et al*., 1995). In the current study, the increase ACE inhibition by cheese and yogurt in the presence of *A. sativum* and/or FC during storage may be explained by the increase bioactive peptides released from milk through the proteolytic activities of LAB (Gobbetti *et al*., 2000; Pihlanto-Leppala *et al*., 1998). Several studies showed relationship between proteolysis and ACE inhibitory activities in milk products such as Meisel *et al*. (1997) showed that inhibitory activity of ACE increased with proteolysis increased during cheese ripening but when the proteolysis exceeded a certain level the inhibition of ACE activity decreased. In addition, this relationship have been showed also in Parmesan cheese (Addeo *et al*., 1992), Italian cheeses (Smacchi and Gobbetti, 1998), enzyme-modified cheeses (Haileselassie *et al* 1999), Manchego cheeses (Gomez-Ruiz *et al*., 2002), sheep milk yoghurt (Papadimitriou *et al*., 2007) and soy yogurt (Donkor *et al*., 2005). In the present study, ACE inhibition activity was found to be highly correlated with proteolysis activities which ranged 70-87% for yogurt and 94-99% for cheeses. However, plain cheese had very low correlation (1%), this may have occurred because of the potent bioactive peptides have been broken down further to less active smaller peptides and free amino acids (Pripp *et al*., 2006). The low correlation in plain cheese suggested that, the formation of bioactive peptides from milk proteins were enhanced in the presence of fish collagen, *A. sativum* and the combination of the two. In fact, bioactive peptides with ACE inhibitory activity have been found in collagen from various fish species including shellfish, tuna, bonito, salmon and sardine (Qian *et al*., 2007; Fujita and Yoshikawa, 1999; Matsufuji *et al*., 1994; Ono *et al*., 2003; Yokohama *et al*. 1992). *A. sativum* extracts as well as have reported to possess antihypertensive effects (Isensee *et al*., 1993; Sharifi *et al*., 2003; Hosseini *et al*., 2007). However, lower ACE inhibition activities by fresh cheeses containing *A. sativum* or *A. sativum* and FC than plain cheese could potentially occur due to the removal of bioactive peptides upon binding to plant phenolic compounds (Alexandropoulou *et al*., 2006; Argyri *et al*., 2006; Cilla *et al*., 2008) which were subsequently lost during pressing of cheese in the moulds. Further study need to be carried out to isolation and identification of bioactive peptides responsible for the high ACE-inhibitory activity in both yogurt and cheese in the presence of *A. sativum* and/or FC in order to introduce functional yogurt and cheese with antihypertensive effects that possibly can contribute to lowering blood pressure in people with hypertension.

CONCLUSION

This study showed that the inclusion of *A. sativum* in yogurt and cheese enhanced proteolysis of milk protein. However, this proteolytic activity increases upon addition of fish collagen in both yogurt and cheese. The enhanced proteolytic activity might have contributed to the observed ACE inhibition activity which is found to be more in cheese than in yogurt, not to mention that contributions from other metabolites should not be ignored. The development of yogurt and cheese containing higher concentration of released bioactive ACE inhibitors may deliver health benefits to consumers with hypertension.

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